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A combined nutritional and immunological intervention to activate natural cytotoxicity against breast cancer cells in vitro and in vivo

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14. ABSTRACT Enter a brief (approximately 200 words)
The hypothesis of this Idea Award is that a combination of nutritional and immunological treatments may be effective in stimulating the body's natural immune defenses against breast cancer. Specifically, the project is examining whether retinoic acid (RA), a metabolite of vitamin A shown to modulate the differentiation and/or activation of several types of immune cells, and α-galactosylceramide (α-GalCer), a synthetic lipid known to alter immune function and to exert antitumorigenic activity, will reduce the growth and/or stimulate natural immunity against 4T1 tumors in adult female Balb/C mice inoculated with 4T1 syngenic breast tumor cells. Natural immunity might help to fight the tumor. In year 3, we have injected 4T1 breast tumor cells intravenously to simulate hematogenous diffusion and have examined the immune system response, micro-metastases, and the expression of Matrix Metalloproteinase (MMP) genes that may be involved in the metastasis to the lungs. We found that in vivo treatment with RA and αGalCer reduced the number of micro-metastases, and reduced expression of certain MMP mRNAs, especially MMP3. Treatment also resulted in systemic changes in immune system cells which included enrichment of CD11b-positive myeloid cells, of which granulocytes (GR1-positive cells) are the dominant type. The results to date support the idea that a nutritional and immunological combination treatment in vivo could be effective in reducing the growth and metastasis of breast tumor cells.

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Introduction

This is a year 3 annual report for the project: **A combined nutritional and immunological intervention to activate natural cytotoxicity against breast cancer cells in vitro and in vivo**.

In our original grant proposal we hypothesized that combining retinoic acid (RA), a metabolite of the essential nutrient vitamin A, with immune stimulation using alpha-galactosylceramide (αGalCer), a synthetic lipid known to alter immune function and to display antitumorigenic activity in vivo, could stimulate the immune system against breast tumor cells (1). The central hypothesis of this Idea Award is that a combination of nutritional and immunological treatments may work together to stimulate the body's natural immune defenses against breast cancer. The conceptual model for our studies is shown in Figure 1. Our hypothesis was based on *1)* the work of others showing that αGalCer, a ligand for CD1d, an MHC-I like receptor, has potent antitumor effects against the growth of melanoma cells, lung cancer cells (1) and other tumors in several animal models, and *2)* our own research showing that RA can induce the expression of CD1d on human and murine monocytic cells (2). We thus proposed that the combination might be an effective way to stimulate the body's immune defenses, including dendritic cells (DC) and natural killer (NK) cells against tumor cells in vivo. The idea was relatively high risk but considered novel, and the benefits, if shown, could be considered for clinical application.

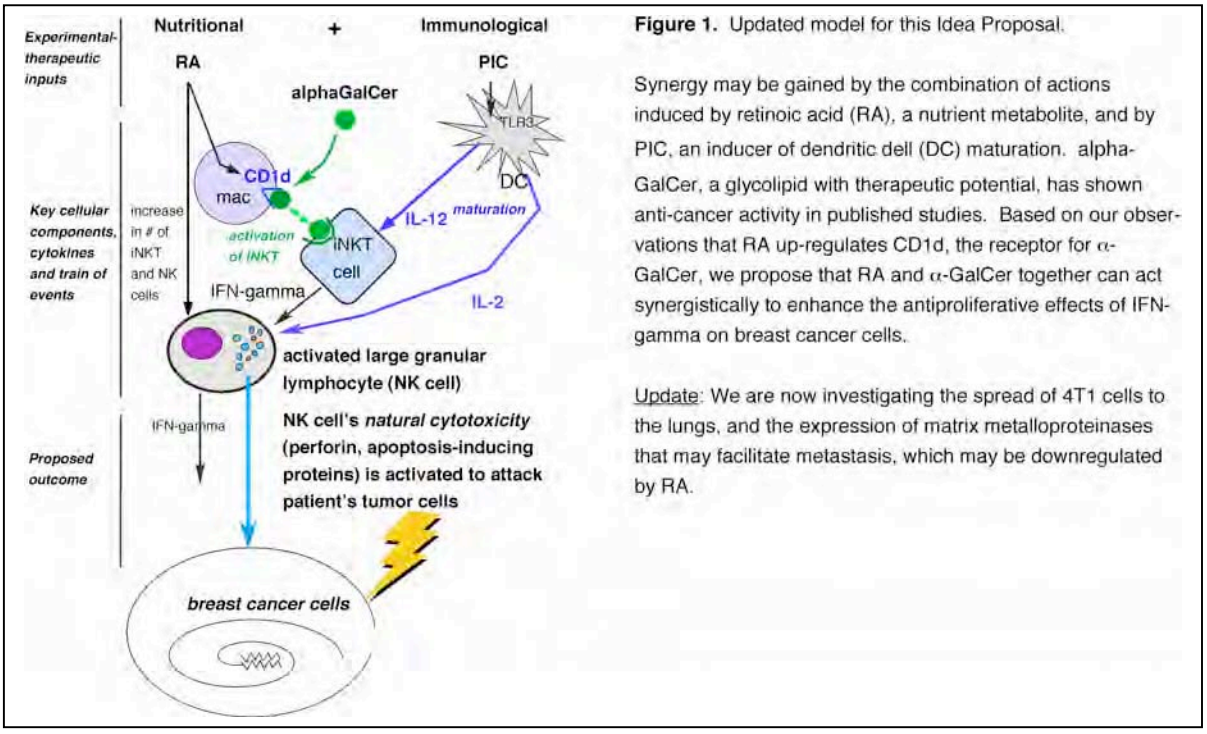

Figure 1. Updated model for this Idea Proposal.

Synergy may be gained by the combination of actions induced by retinoic acid (RA), a nutrient metabolite, and by PIC, an inducer of dendritic dell (DC) maturation. alpha-GalCer, a glycolipid with therapeutic potential, has shown anti-cancer activity in published studies. Based on our observations that RA up-regulates CD1d, the receptor for α-GalCer, we propose that RA and α-GalCer together can act synergistically to enhance the antiproliferative effects of IFN-gamma on breast cancer cells.

Update: We are now investigating the spread of 4T1 cells to the lungs, and the expression of matrix metalloproteinases that may facilitate metastasis, which may be downregulated by RA.

Body

Previously we have shown that RA increases the expression of CD1d on monocytic cells and macrophages. CD1d is expressed by several types of cells throughout the body, particularly by antigen-presenting cells, such as DC. CD1d is upregulated transcriptionally by RA in THP-1 monocytic leukemia cells, a model for human monocytes, and murine macrophages (2). CD1d is known to be a direct factor in the stimulation of natural killer T cells, referred to as NKT cells, invariant NKT cells, CD1d-dependent NKT cells, or Vß8-expressing NKT cells (3-5).

As an animal model, we are using female Balb/c mice, and as a breast cancer model we are using the 4T1 syngeneic tumor cell line, which was originally isolated from Balb/c mice (6).

One of our main findings from year 2 was that the combination of RA and αGalCer did not act directly to kill 4T1 cells in vitro. As shown in Figure 2 of last year's progress report, we tested whether RA and αGalCer directly inhibit 4T1 cell growth in vitro by culturing 4T1 cells in the presence and absence of RA (20 nM) and αGalCer (100 nM) for 24 hours. DNA synthesis was measured, first by ^{3}H-thymidne incorporation and scintillation counting, and second by measuring the distribution of 4T1 cells in the G1, S and G2/M phases of the cell cycle, which would detect changes in S-phase cells. No differences were detected in either assay. Thus, this result indicated the inhibition is not due to an action of these compounds on the tumor cells directly, which then focused our attention on how the actions of these compounds might be mediated through other cells.

Another finding from year 2 was that injection of 4T1 cells resulted in significant splenomegaly, also reported by others (7). We showed that there is significant correlation between spleen weight and tumor weight.

Additionally, we examined mice injected in the mammary fat pad for metastasis of 4T1 cells to the lungs. For this assay, we digested the lung tissue and cultured single cell suspensions from each mouse in medium with 60 µM of 6-thioguanine, which suppresses the growth of lung cells but not of the 4T1 tumor cells, and then after 14 days of culture we fixed and stained the surviving tumor cells with methanol and 0.03% methylene blue dye for counting. The results showed that injection of pulsed DC resulted in a considerable reduction in the 4T1 cell colony number, suggesting a decreased rate of metastasis. These results thus formed the background for the experiments we have conducted in year 3.

The following section summarizes our work over the 3 years and presents results for year 3 in detail.

Summary of Year 1 and 2 results

Year 1

1-1. MRI imaging was tested, but considered no more effective while more expensive than morphological analysis. We had proposed to use a new instrument at Penn State to image the tumor cells in vivo, a procedure that we thought would allow for multiple measurements over time. The Small Animal MRI facility had just opened and the methodology had not been applied previously for the purpose of detecting breast tumors. Several mice were injected with 4T1 breast cancer tumor cells, which we obtained from Dr. Danny Welch, University of Alabama, Birmingham. We conducted MRI one week, and again 10 days and 14 days after tumor cell inoculation into the inguinal fat pads of Balb/C mice. Initially, we could not visualize the tumor tissue by MRI, and even the distinction of major visceral organs was not as clear as we had hoped. The mice had to be anesthetized with isoflurane for a long period, up to an hour, to obtain good images, and the duration of the anesthesia, which varied among mice, was a concern to us. After the tumors had grown to a palpable size, then they also were detectable by MRI. But since the purpose of using the MRI was to obtain early and sequential measures, we decided that the standard procedure of palpation and tumor measurement, using an accurate digital caliper that we purchased, would be just as effective.

1-2. RA regulated CD1d/αGalCer-mediated NKT cell functions. We began by demonstrating that RA and αGalCer, added to cultured spleen cells for up to 3 days, affected immune cell populations. A-GalCer induced, and RA regulated αGalCer-induced, production of IL-4 and IFNγ mRNA, (measured by real-time RT-PCR) and protein secretion (measured by ELISA using cell supernatants) collected on day 3. RA also regulated the α-GalCer-induced expression of intracellular cytokines (IL-4 and IFNγ) determined by flow cytometry on day 3 (2).

1-3. RA was effective in reducing NKT tumor cell growth. We tested the ability of RA to alter growth of tumor cell lines, using two NKT tumor cell lines, DN32.2 and TCB11. RA significantly reduced the rapid proliferation of these cells. Proliferation was also decreased in co-cultures of NKT cells with DC, but not with total spleen cells.

1-4. αGalCer stimulated cytokine production of spleen cells. To pretest the ability of splenic DC to function in our model, we tested spleen cells for production and secretion of IL-4 and IFNγ. These cytokines were produced only when cultured with αGalCer. The presence of the mouse NKT tumor cells, especially TCB11, further increased spleen cell cytokine production, although these cells alone produced neither cytokine.

1-5. Cytokine production by NKT cells was regulated by RA and αGalCer. The production of IL-4 and IFNγ by spleen cells was differentially regulated by RA, as RA

increased the output of IL-4 when αGalCer-activated spleen cells were co-cultured with NKT cells, but at the same time RA reduced the output of IFNγ.

Results from these initial studies were presented at the Era of Hope meeting, see abstract under Reportable Outcomes.

Year 2

2-1. RA and αGalcer did not directly affect 4T1 cell growth. Because in our model we planned to treat the APC (DC) with RA to induce CD1d and with αGalCer as an activator of iNKT cells, we needed to know whether these agents directly affect the growth of 4T1 cells, or whether the effect if any is more likely due to the mediation of treated DCs. Therefore we tested whether RA and αGalCer directly affect 4T1 cell growth in vitro. As reported on page 6, treatment with RA and αGalCer did not change the cell proliferation rate as measured by ^{3}H-thymidine incorporation. This was confirmed by cell cycle distribution analysis, which also did not show any difference in the percentage of cells in G, S, or G2/M phases of the cell cycle among the different treatments. These data suggest that even though RA and αGalCer have both been shown to reduce the growth of some tumor cells, they do not have a direct effect on the growth of the 4T1 breast tumor cells in vitro. From these results we can conclude that if changes in the growth of the 4T1 breast tumor cells are observed with RA and αGalCer treatments in vivo, they will be most likely to be mediated through activation of other cells and not directly on the growth of 4T1 cells.

2-2. RA and αGalCer treated DC reduced the 4T1 cell growth and metastasis in vivo. Dendritic cells were isolated from spleens of adult Balb/c mice, treated in vitro with RA and αGalCer, and administered by the i.v. route, at the time of the 4T1tumor inoculation which was given subcutaneously (s.c.) into the right mammary fat pad. At the time of dissection, day 26, tumors in nearly all of the mice were well localized as tight nodule(s) without local invasion. Body weight as well as liver and lung weight did not differ among the treatment groups; however, a marked difference was noted regarding spleen size and weight. With the injection of RA-treated, αGalCer pulsed DCs, the spleen weight was reduced although not back to the normal level. The same pattern was observed for tumor weight, implying that growth was slowed. The correlation between spleen and tumor weight was significant (R^2 = 0.56, P<0.05).

2-3. 4T1 cells in the lungs were reduced by in vivo treatment with RA- and αGalCer-treated DC. After 14 days of culture to allow 4T1 colonies to grow from dispersed lung tissue, the 4T1 cell colonies were fixed with methanol and stained with methylene blue dye for counting. Injection of pulsed DC resulted in a considerable reduction in the 4T1 cell colony number, suggesting a decreased metastasis rate in the RA+αGalCer treated mice.

Key Research Accomplishments in Year 3

In the annual report for year 2, we reported that 4T1 breast tumor cell inoculation of Balb/c mice, using the orthotopic model in which tumor cells were injected into the mammary gland fat pads, caused systemic changes. Along with the local tumor growth, the spleen was greatly enlarged, with myeloid cell enrichment determined by flow cytometry, and lung metastases observed by tumor cell colony growth ex vivo. The results demonstrated the potential effectiveness of RA and αGalCer pulsed-DC to inhibit primary tumor growth and lung metastasis.

Nonetheless, neither RA nor αGalCer affected 4T1 cell growth and cytokine expression during in vitro culture experiments. This implied that the in vivo results are not due to a direct cytostatic effect or RA or αGalCer on these cells. We therefore proposed that RA and αGalCer might exert their regulatory effect through the regulation of the host immune response. A second concern caused us to modify our model. Through year 2, we had injected 4T1 cells into the mammary fat pad. Although we observed tumor growth, it was not consistent from mouse to mouse. We generally observed a significant reduction in tumor weight due to injection of DC, which was significant in pooled experiments, but the treatment groups (vehicle, RA, αGalCer, RA+αGalCer) were not different from each other, due to variation. We also observed metastasis to the lungs, in some animals in our 26-day study. A problem, however, was that by this time the mammary tumor size was large and we could not extend the study, due to having reached end-point criteria for euthanasia as defined in our IACUC proposal (palpable tumor > 1 cm external diameter) in some of the mice. Moreover, the splenic response was quite pronounced by day 26.

In the literature, various means of delivering 4T1 cells have been described, from injection into the mammary gland fat pad, to intrascapular injection, to intravenous injection. The latter route, used by Kim et al (8) for 4T1 cells mimics the hematogenous spread of cells from the tumor site through the blood to other organs. With this route of tumor cell delivery, a much smaller number of tumor cells is delivered, and metastasis to the lungs is measured after approximately 12 days using methods to determine micro-metastasis (histology and growth of 4T1 colonies from the lung tissue). In year 3, we modified our IACUC protocol (now Penn State IACUC Approval #33527) in order to test this model of intravenous delivery of 4T1 cells as a way to mimic the spread of breast tumor cells through the circulation to the lungs. Results from year 3 including these studies are reported here, focused on:

1) Immune cell populations, since these were observed to change in the mammary fat pad inoculation model;

2) Lung metastases measured by counting and by extracting the crystal violet dye that stains the tumor cells for spectrophotometry;

3) Matrix Metalloproteinase (MMP) gene expression. MMPs are implicated in the spread of tumor cells (9). We screened several MMP genes and then focused on MMP3 and a membrane type, MT-MMP1.

3-1. Spleen changes were moderate after intravenous injection of 4T1 cells (SOW Tasks 15, 16 17)**.**

4T1 cells were injected intravenously ($5x10^4$ cells in 100 µl of PBS). Mice were then separated into four groups for treatment, which began immediately after inoculation. Retinoic acid was given orally, 37.5 µg/mouse/day daily; αGalCer was injected, 2 µg/mouse, s.c., every other day; canola oil was used as vehicle control, and βGalCer was used as control for αGalCer. On day 12, mice were euthanized for analysis. As shown in Figure 2, overall spleen weight stayed similar to the no-tumor control. This contrasts sharply with our results in the 26-day studies in which spleen weight was nearly 3 times, and reduced about 25% by injection of DC (shown in year 2 progress report). Thus, these results were different compared with the orthotopic model we had used previously.

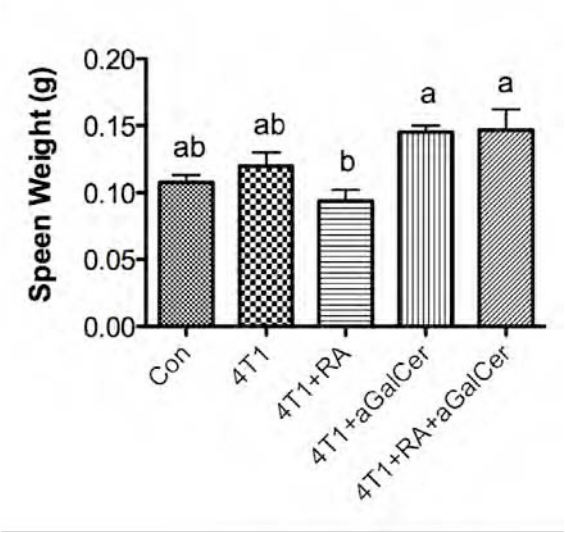

Figure 2. Spleen weight in Balb/c mice 12 days after 4T1 cell injection. Bars that do not share a letter were significantly different, *P*<0.05 (one-way ANOVA).

In the 12-day study, we also conducted an analysis of the spleen cell populations, as shown in Figure 3. Twelve days after injection of 4T1 cells, there was no change in the proportion of CD3, CD4, and CD8 positive T cells, TCRß (NKT cells), NK cells, and CD19 B cells, nor was there an effect of RA or αGalCer treatment on these populations. However, there was a near doubling of CD11b-positive cells, indicating a greatly increased myeloid cell population, shown in the lower panel. This change is similar to our previous observations in the orthotopic model. It also indicates that this change occurs early in response to the 4T1 tumor cells. Among the myeloid cells lineage, CD11b monocytes and Gr1-positive granulocytes comprise the major populations. There were relatively few macrophages or DC in any treatment group. We did not observe any difference between the treatments in 4T1-injected mice under the current conditions. Although there were increased Gr1+ cells in all 4T1-tumor bearing mice, and this is a cell type recently implicated in protumor activity (10), nevertheless the percentage was lower than in our previous studies using intramammary fat pad inoculation of tumor cells. This may have been due to the shorter time of this study.

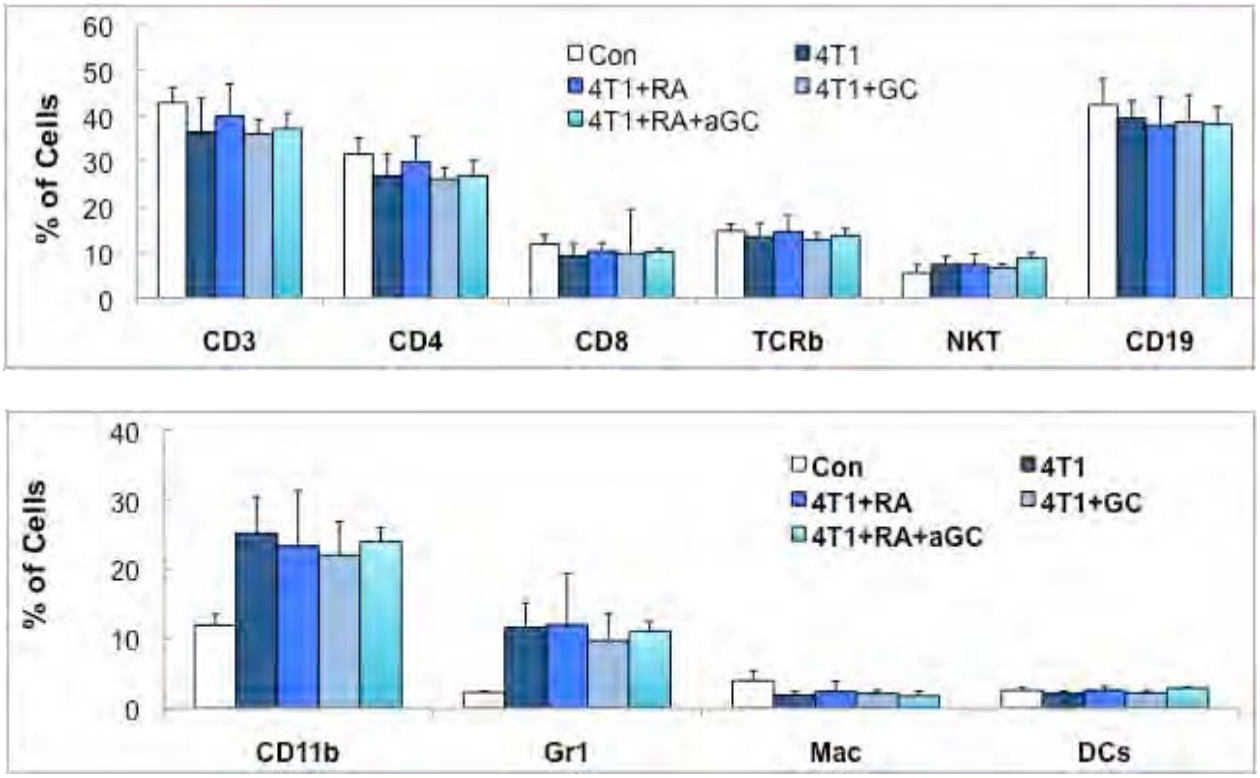

Figure 3. Spleen cell population analysis on day 12 in Balb/c mice inoculated i.v. with 4T1 breast tumor cells. Mouse splenocytes were isolated and subjected to flow cytometry study. The difference between Control (Con) and groups treated with 4T1 cells was significant for CD11b and Gr1-positive cells, but there were no differences due to RA or αGalCer treatment for any of the populations.

3-2. RA and αGalCer inhibited lung metastasis (SOW Task 18)

Hematoxylin and eosin (H&E) staining of the lung tissue showed that mice inoculated with 4T1 tumor cells but without any in vivo treatment with RA or αGalCer after inoculation had multiple large tumor foci, while in mice that received these treatments the number and size of the foci were reduced. Figure 4 showed representative images from tumor-injected mice after 4T1 injection without treatment (Fig. 4, left) and with RA and αGalCer treatment (Fig. 4, right).

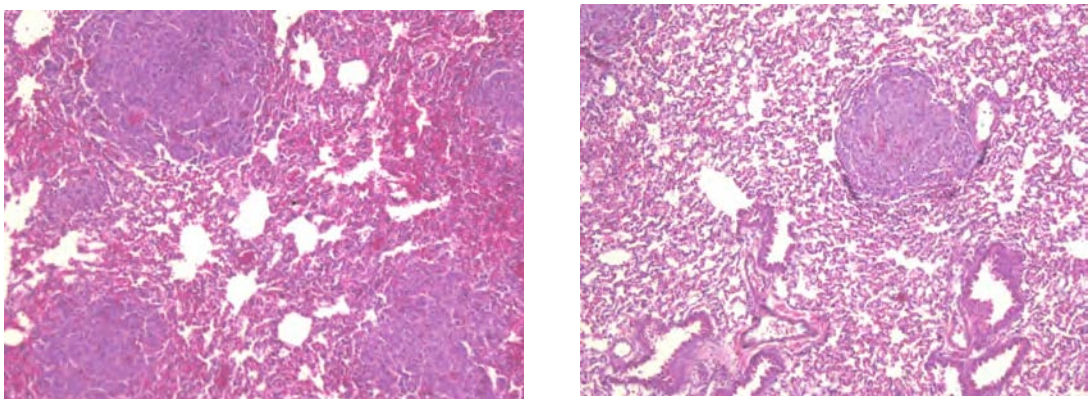

Figure 4. H&E staining of mouse lung tissue. Left, Representative histology from 4T1 group without treatment. The field shows 4 metastatic growths and limited normal tissue; right, representative histology of 4T1 injected mice treated with RA and αGalCer. The filed shows one metastatic growth, with significant areas of normal alveolar structure. Both samples were taken 12 days after tumor cell injection.

To quantify the metastasis rate, lung tissue was digested with elastase and collagenase IV to isolate a single cell suspension (11). Cells were plated in 6-well plates to test the in vitro tumor foci formation. Cells were cultured with 6-thioguanine to eliminate normal lung cells, and after 14 days of culture the 4T1 cell colonies were counted. As shown in Figure 5, left, direct counting of the foci showed that all of the in vivo treatments had significant efficacy in reducing the number of micro-metastases in the lung ($P<0.05$). The counting data were confirmed when we washed the plates and then dissolved the methyl blue dye used for detection and measured the optical density of the extract at 570 nm (Figure 5, right).

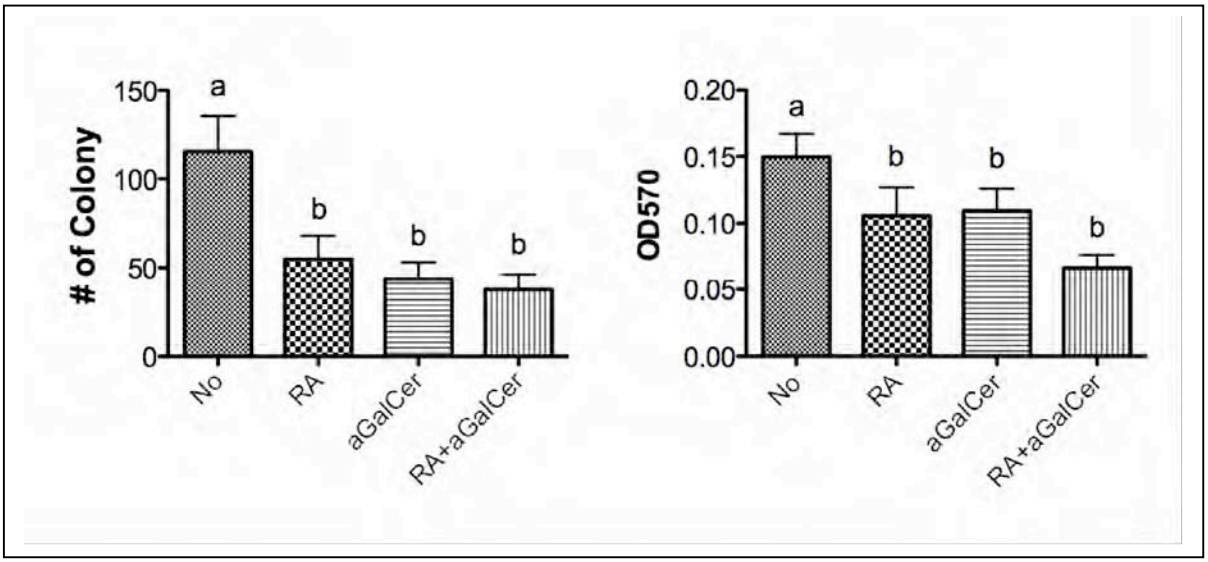

Figure 5. Colony counts of 4T1 cells in 4T1-inoculated mice treated with RA, αGalCer, or both in combination. Lung tissue was collected on day 12 and lung cells were cultured for 14 days (see text). Colony growth was significantly reduced by all treatments (left), and the OD570 reading, representing dye associated with the tumor cells (right), was also reduced. a>b, $P<0.05$.

3-3. RA decreased the expression level of MMPs in lung tissue (SOW Task 19, modified from analysis of IRF-1 to MMPs)

Having observed reduced metastases, we have begun to explore possible mechanisms. Metastasis is extremely complicated but proteases are clearly involved. Matrix metalloproteinases (MMPs) play important roles in tumor invasion and metastasis (2). Some publications have suggested that RA can modulate MMP gene expression. We first tested the gene expression levels of several MMPs, including MMP2, MMP3, MMP9, and MT MMP-1, in lung tissue by real-time qRT-PCR. From this screening, the membrane-type MMP, MT-MMP1, and MMP3 were expressed and regulated in the lung tissue collected in mice in our previous experiments, and thus MMP3 and MT-MMP1 were selected for analysis in the lung tissue of mice in our 12 day study, after 4T1 cell inoculation and treatment with RA, αGalCer or both in combination. As shown in Figure 6, the lungs of mice with 4T1 cells had much higher levels of expression of MT-MMP1 and MMP3 mRNA, indicating that the tumor alone activated the expression of these genes. This would be consistent with tumor-induced promotion of metastasis (12). Also shown in Figure 6, treatment with RA significantly decreased the levels of MMP3, with a similar tendency for MT-MMP1. αGalCer did not affect gene expression, nor did it modify the effect of RA in the combination treatment. The lower levels of MMPs could help to explain the reduced metastasis rate in RA-treated animals. These assays need to be replicated in a repeat experiment for confirmation.

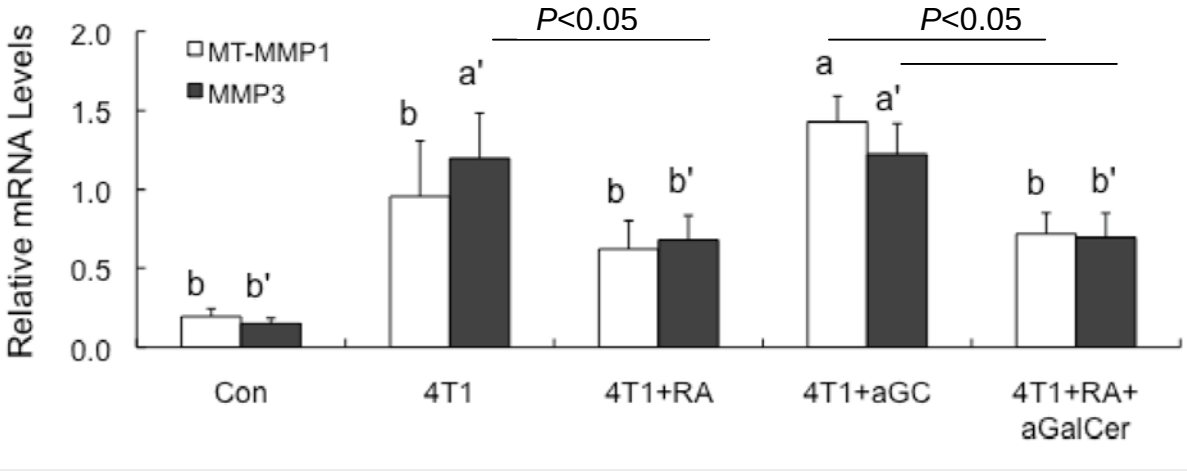

Figure 6. RA inhibited the expression of the matrix metalloproteinases MT-MMP1 and MMP3 mRNA in mice after 4T1 injected with 4T1 cells. Groups with different letters, a>b for MT-MMP-1, a'>b' for MMP3, were significantly different, *P*<0.05.

Discussion

In the current model, we have shown that 4T1 breast tumor cell injection caused tumor formation in the lungs of mice within a short period of time (12 days). It also resulted in moderate systemic changes in immune system cells which included enrichment of CD11b-positive myeloid cells, of which granulocytes are the dominant cells. Animals treated with RA and αGalCer exhibited a reduced rate of lung metastasis as demonstrated by histological staining as well in our micrometastasis cell culture assay. A decreased espression level of certain MMPs, especially MMP3, was observed, which could help to explain the mechanism through which RA decreases the spread of 4T1 cells to the lungs.

The basic idea proposed in this IDEA award was that DC treated with RA and αGalCer may be able to suppress breast tumor growth, or that RA and αGalCer might be effective when administered directly to mice in vivo, after tumor inoculation. Our study has found some support for an effect of DC, but we did not observe consistent differences due to pretreatment of the DC with RA or αGalCer. Thus, treatment with autologous DC appears to be promising. We are not yet convinced that RA and αGalCer were inactive, as some of the treated mice had smaller tumors. However, overall the variability in the data precluded drawing the conclusion that pretreatment of the DC with RA and αGalCer ex vivo had a significant effect on tumor growth in vivo. 4T1 is known to be an aggressive tumor. Nevertheless, while the

tumor grows rapidly, it remains quite local and encapsulated when it is injected into the mammary gland fat pad. By the time that lung metastases were apparent in our studies, the mammary tumor was of such a size that mice had to be euthanized. We therefore decided to test a hematogenous model in year 3. The hematogenous model proved more tractable, and the reduced number of tumor cells in the lungs as shown in Figure 4 and Figure 5 is quite encouraging. This study needs to be repeated (as planned as SOW 20 of our original proposal) and we have applied for a no-cost extension to allow this task to be completed and these results to be confirmed. The finding that MMP3 mRNA is significantly reduced by RA is also encouraging, with MT-MMP1 tending in the same direction. RA could influence metastasis to the lungs by limiting the tumor-induced expression of these genes. αGalCer did not have the same effect, nor did it interact with RA. Future studies should be conducted to confirm whether RA represses MMP3 expression, determine the mechanism, and whether other MMPs are similarly affected in the lungs or other tissues (liver) of 4T1 cell-injected mice.

Clearly, the Balb/c mice that received the 4T1 cell tumors, whether by the intra-fat pad (orthotopic) or the hematogenous route, responded immunologically to the tumor cells. This is noteworthy because this is syngeneic model in which direct immunological rejection of the tumor cells is not expected, but the increase in spleen weight, and increase in CD11b+ and Gr1+ monocytic cells, indicates that there is a significant immune activation induced by 4T1 cells. The significance of these changes needs further evaluation. Recently, Yan et al. (10) reported that CD11b-positive Gr1+ granulocytic cells can function in a protumor manner. Clearly these cells are increased by the presence of the 4T1 tumor cells, as they were low in control mice. We did not observe changes in NK cell number, which we had hypothesized, but it is possible that NK cell activation occurred, and this still needs to be investigated.

Overall, while the results do not fully confirm our initial hypothesis, they supports parts of it. The results do point to DC as having a potential benefit, and they indicate that RA and αGalCer given in vivo can play a modulating role on tumor growth and particularly on the spread of tumor to the lungs. Since metastasis is the most frequent cause of breast cancer mortality, it is very important to find treatments that prevent or slow the rate of metastases and tumor growth after metastasis.

The treatments with RA and αGalCer were very well tolerated, with no reduction in body weight and similar body condition in all treatment groups. Thus, RA and αGalCer could be useful as adjuvants. Adjuvant treatments that stimulate the immune system to fight tumor cells and reduce metastases could potentially be effective against many types of cancer, and thus they are worth pursuing further. We hope to finalize the current set of observations with another study using the hematogenous route of delivery, and to complete studies on the regulation of MMP3 expression in the lungs of mice, with and without 4T1 tumors, and in mice with and lacking the CD1d gene.

Plan for completion of studies during no-cost extension (August 1, 2010 – July 31, 2011)

1. Conduct SOW 20 and 21: Increase sample size in 12-day study using i.v. injection of 4T1 cells,

A. Measure rate of lung metastases (colonies and colony-associated dye after in vitro culture with 6-thioguanine.

B. Measure lung tissue MT-MMP1 and MMP3 mRNA to confirm observations and increase statistical power of analysis.

2. Test dependence of results on CD1d expression. Our hypothesis was based in part on observations that RA increased CD1d expression, which could then bind αGalCer and stimulate NKT cells. This can now be tested definitively by conducting the 12-day metastasis experiment described in year 3 in CD1d-null compared to CD1d-expressing mice.

A. Order CD1d-null mice on Balb/c background (Jackson Laboratory) and Balb/c-J mice as wild type control.

B. Treat in vivo with 4T1 cells ($5x10^4$/mouse) using the hematogenous model as in year 3. Treat mice in vivo with RA and αGalCer. After 12 days, harvest lungs and spleen.

C. Conduct lung histology (as in Figure 4)

D. Conduct quantification of lung micrometastasis using dye assay (as in Figure 5).

E. Measure lung tissue MT-MMP1 and MMP3 mRNA to confirm observations and increase statistical power of analysis (as in Figure 6).

F. Analyze spleen cells by flow cytometry (as in Figure 3)

G. Analyze results as in figures 2-6 of this report.

H. Prepare manuscript and report final results (complete Milestone 8).

Reportable outcomes

We presented data in abstract form at the Era of Hope meeting in Baltimore in 2008. A copy of our Era of Hope abstract is as follows:

Retinoic acid and alpha-galactosylceramide, a ligand for CD1d on antigen-presenting cells, differentially regulate the production of immunoregulatory cytokines by cultured dendritic cells and splenocytes.

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Background and objectives: Activation of the natural immune system is promising as a way to inhibit tumor growth. Dendritic cells (DC), natural killer (NK) cells, and a specialized subset of T cells known as iNKT cells are among the cell types likely to inhibit tumor growth in vivo. Based on previous results, we postulated that a combination of retinoic acid (RA), an agent that often inhibits cell growth and induces cell differentiation, and which can induce the expression of CD1d by antigen-presenting cells, may augment the activation of iNKT cells in the presence of α-galactosyl-ceramide (αGalCer). In animal models, αGalCer has shown encouraging results against several types of cancer, but to our knowledge it has not yet been tested in combination with RA or cytokine-inducing agents such as poly-I:C, a strong inducer of interferons. We thus have proposed that a triple nutritional-immunological combination of RA, αGalCer and poly-I:C could be effective for breast cancer prevention, based on 1) the ability of RA to induce CD1d, 2) of αGalCer to bind to CD1d on antigen-presenting cells (dendritic cells, DC, or macrophages) and activate iNKT cells, and 3) of poly-I:C to stimulate the production of cytokines, especially IFNs, which also activate NK cells. We plan to test the basic concept that these agents may synergistically inhibit tumor growth, in cultured cells and in mice in vivo. In the first 6-month period of our award, our objective was to test whether αGalCer combined with RA can regulate the proliferation of cultured DC and splenocytes, and their production of immunoregulatory cytokines.

Methods
DC were prepared from the bone marrow of the femur and tibia of adult (> 8 wk) female Balb/c or C57BL/6 mice. The cytokine GM-CSF was added to the cultures in complete media every 3 days for total of 9 days to induce growth of immature DC. On day 9, TNFα (5 ng/ml) was added to induce DC differentiation. Splenic mononuclear cells were treated with RA ± αGalCer for 24 hours. To monitor NKT cell proliferation, differentiated DC or isolated splenocytes in 96-well plates were treated with RA (20 nM) or αGalCer (100 nM) for 24 h. The inactive anomer, β-GalCer (100 nM), was used as control. Two NKT cell lines, as potential responders to the presentation of αGalCer bound to CD1d, were then added to the DCs or splenocytes for 48 h. For the last 4 hr, 3H-thymidine was added to monitor cell proliferation.

Results to date

In our preliminary studies:

1. RA significantly reduced the rapid proliferation of the two NKT tumor cell lines, DN32.2 and TCB11. Proliferation was also decreased in co-cultures of NKT cells with DC, but not with spleen cells.
2. Spleen cells produced and secreted IL-4 and IFNγ only when cultured with αGalCer. The presence of the NKT tumor cells, especially TCB11, further increased spleen cell cytokine production, although these cells alone produced neither cytokine.
3. The production of IL-4 and IFNγ by spleen cells was differentially regulated by RA, as RA increased the output of IL-4 when αGalCer-activated spleen cells were co-cultured with NKT cells, but at the same time RA reduced the output of IFNγ.

Conclusions: The growth-inhibitory effects of RA against the NKT tumor cells is encouraging, but further in vivo studies are needed. αGalCer markedly induced IFNγ production by splenocytes. The attenuation by RA needs to be further evaluated, especially with PIC included in the triple stimulation model.

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A ms reporting the results comparing the routes of 4T1 cell injection on spleen cell and immune cell response, lung tumor metastasis, and MMP gene expression is in progress. We would like to repeat the studies as described above before finalizing the manuscript.

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